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Prevention and Treatment of Neurofibromatosis Type 1-Associated Malignant  
Peripheral Nerve Sheath Tumors

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14. ABSTRACT The most common cause of death in Neurofibromatosis Type 1 (NF1) patients is malignant peripheral nerve sheath tumor (MPNST). MPNSTs are aggressive Schwann cell-derived neoplasms that typically arise from precursor lesions such as plexiform neurofibromas. Although gross total resection of MPNSTs is potentially curative, this occurs in only a small minority of cases. Radiotherapy and chemotherapy have almost no effect on patient mortality. NF1 patients have an approximate 10% lifetime risk of developing an MPNST. Thus, development of safe and effective MPNST preventative therapies could have an important impact on NF1 patient morbidity and mortality. In this grant, we are testing the hypothesis that chronic administration of agents that promote apoptosis and/or inhibit pro-survival autophagy will inhibit MPNST formation and progression in transgenic mouse models of MPNST. Specifically, we are examining the mechanisms of action and <i>in vivo</i> utility of two classes of drugs, BH3 mimetics and lysosomotropic agents, on MPNSTs. The drugs that we are testing are approved for human use and could be rapidly advanced into human MPNST clinical trials if our pre-clinical testing yields positive results.					
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**Title of Grant:** Prevention and Treatment of Neurofibromatosis Type 1- Associated Malignant Peripheral Nerve Sheath Tumors

**Award #:** W81XWH-14-1-0073

**Principal Investigator:** Kevin A. Roth, MD, PhD

**Annual Report:** 04/01/2016– 03/31/2017

## **1. Introduction**

Neurofibromatosis type 1 (NF1) has a frequency of approximately one in 3,000 humans and decreases life expectancy by ten to twenty years. Malignant peripheral nerve sheath tumors (MPNSTs) are the leading cause of death in NF1 patients and typically arise from NF1-associated precursor lesions. NF1 patients have an approximate 10% lifetime risk of developing a MPNST and this risk may be as high as 30% in NF1 patients with symptomatic plexiform neurofibromas. MPNSTs afflict NF1 patients in the prime of their lives, median age at diagnosis being approximately 40 years, and have a poor prognosis with median disease specific survival of approximately five to eight years. Gross total surgical resection is the only curative therapy and is unobtainable in the vast majority of patients. Radiotherapy and chemotherapy have proven largely ineffective in extending MPNST patient survival. Tumor formation and malignant progression are both dependent on the ability of tumor cells to evade normal cell death inducing stimuli. Numerous studies have shown that overexpression of anti-apoptotic Bcl-2 family members such as Bcl-2, Bcl-XL and MCL-1 can decrease tumor cell sensitivity to both radiotherapy and chemotherapy. Small molecule inhibitors of anti-apoptotic Bcl-2 proteins which have a functional BH3 domain, so called “BH3 mimetics”, can potentiate tumor cell sensitivity to standard chemotherapeutic agents. Similarly, cytoprotective autophagy is commonly increased in tumor cells permitting these cells to survive in nutrient poor and hypoxic conditions that would kill normal cells. Cytoprotective autophagy can be inhibited by lysosomotropic agents such as chloroquine (CQ) that inhibit lysosome degradation of autophagic vacuoles and their contents. To date, no studies of combined BH3 mimetic and lysosomotropic agents have examined their potential utility as MPNST chemopreventive agents in either animal models or in NF1 patients.

## **2. Keywords**

Apoptosis  
Autophagy  
Lysosomotropic agents  
Bcl2 family members

## **3. Accomplishments**

**What were the major goals of the project?**

1. To determine therapeutic effects of BH3 mimetics and lysosomotropic agents to inhibit Schwann cell hyperproliferation, MPNST formation and progression in transgenic mouse models.
2. To determine the effects and mechanisms of action of BH3 mimetics and lysosomotropic agents, alone and in combination, on NF1 patient-derived MPNST cell lines *in vitro*.

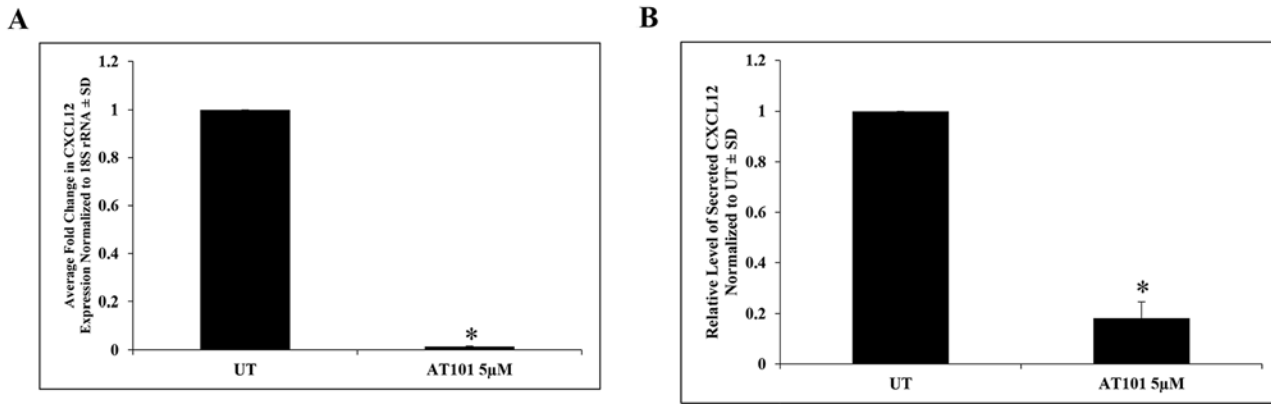
**What was accomplished under these goals?**

Since submission of last year's (04/01/2016 – 03/31/2017) annual progress report, we continued to make progress on the proposed studies described in that report.

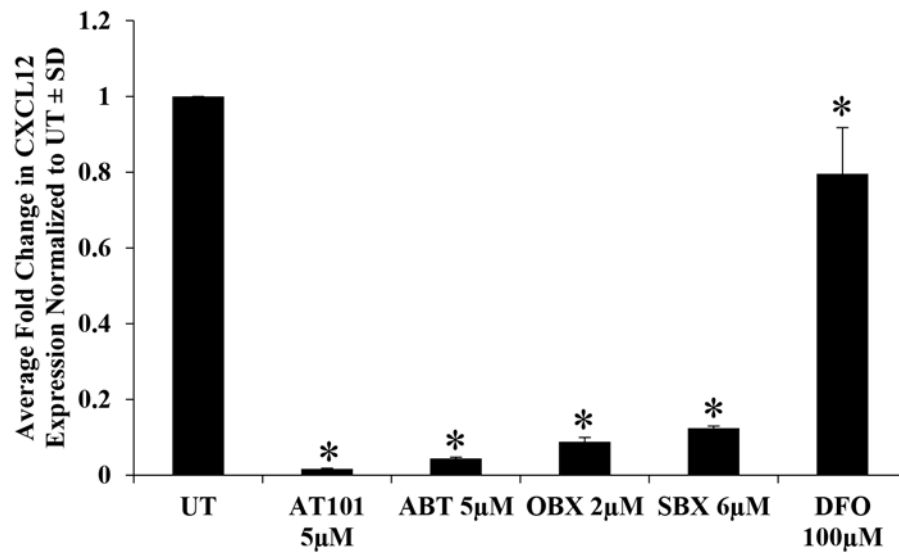
### Key Research Accomplishments:

- We determined that BH3 mimetics suppress CXCL12 expression in MPNST cells *in vitro*.

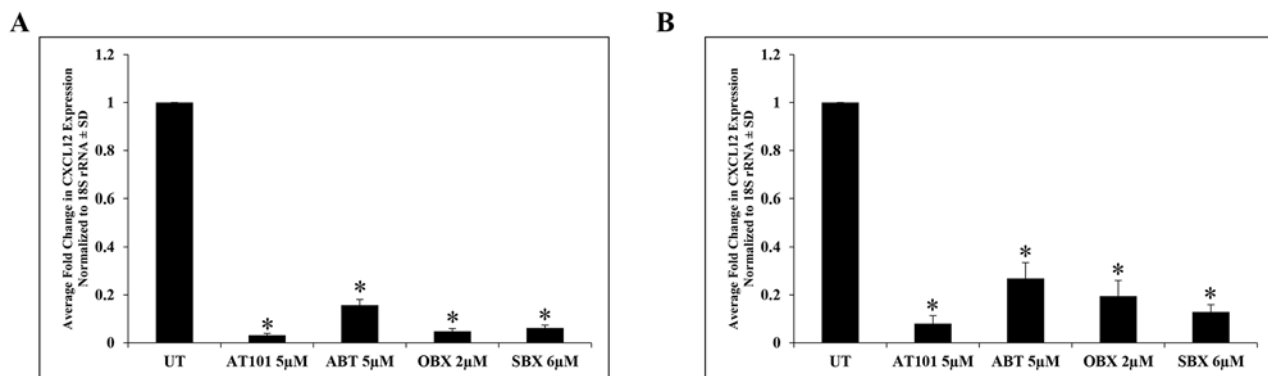
Malignant peripheral nerve sheath tumors (MPNSTs) are aggressive, Schwann cell-derived neoplasms of the peripheral nervous system that have recently been shown to possess an autocrine CXCL12/CXCR4 signaling loop that promotes tumor cell proliferation and survival. Importantly, the CXCL12/CXCR4 signaling axis is driven by availability of the CXCL12 ligand rather than CXCR4 receptor levels alone. Therefore, pharmacological reduction of CXCL12 expression could be a potential chemotherapeutic target for patients with MPNSTs or other pathologies wherein the CXCL12/CXCR4 signaling axis is active. AT101 is a well-established BCL-2 homology domain 3 (BH3) mimetic that we have recently demonstrated to also function as an iron chelator and therefore acts as a hypoxia mimetic. In this study, we found that AT101 significantly reduces CXCL12 mRNA and secreted protein in established human MPNST cell lines *in vitro* (Figure 1). This effect was recapitulated by other BH3 mimetics [ABT-737 (ABT), obatoclax (OBX) and sabutoclax (SBX)] but not by desferrioxamine (DFO), an iron chelator and known hypoxia mimetic (Figure 2). These data suggest that CXCL12 reduction is a function of AT101's BH3 mimetic property rather than its iron chelation ability (Figure 3). Additionally, this study investigates a potential mechanism of BH3 mimetic-mediated CXCL12 suppression: liberation of a negative CXCL12 transcriptional regulator, poly (ADP-Ribose) polymerase I (PARP1) from its physical interaction with BCL-2. These data suggest that clinically available BH3 mimetics might prove therapeutically useful at least in part by virtue of their ability to suppress CXCL12 expression.



**Figure 1: AT101 down-regulates CXCL12 in MPNST cells.** **A.** qRT-PCR analysis of AT101-treated T265-2c cells (5μM, 24h). **B.** AT101 treatment (5μM, 24h) resulted in a significant reduction of secreted CXCL12 protein in T265-2c cells as demonstrated by an ELISA. \**p*-value <0.05.



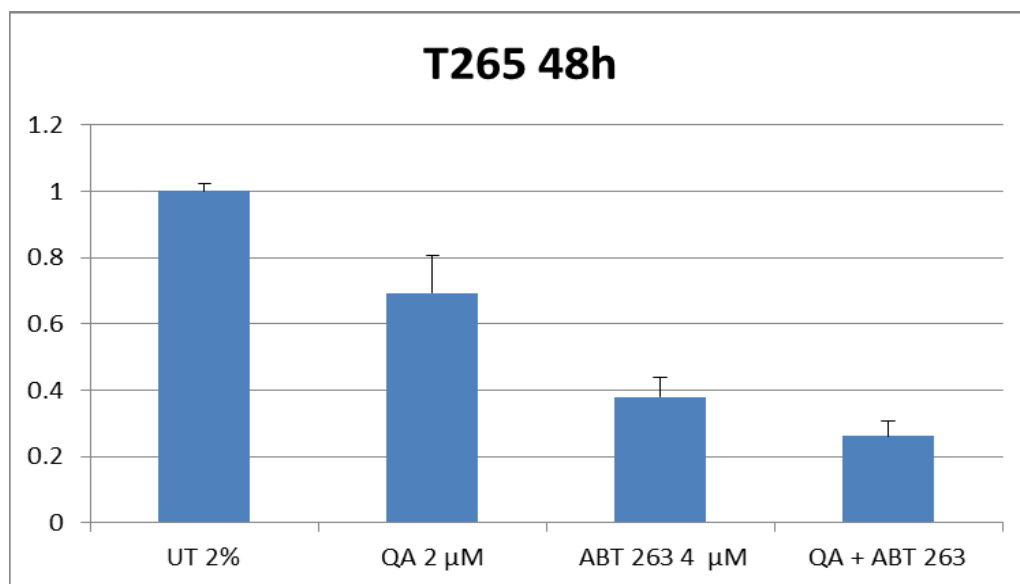
**Figure 2: BH3 mimetics recapitulate the effects of AT101 on CXCL12 expression.** T265-2c cells treated with AT101, ABT, OBX, SBX or DFO significantly suppress CXCL12 mRNA levels compared to no treatment as demonstrated by qRT-PCR. \**p*-value <0.05. Comparison of DFO with AT101, ABT, OBX or SBX treatment resulted in a statistically significant difference in CXCL12 expression (*p*-value <0.01).



**Figure 3: BH3 mimetic suppression of CXCL12 is conserved among multiple MPNST cell lines.** BH3 mimetics suppress CXCL12 mRNA levels in an NF1-derived (90-8) **A.** and sporadic (STS26T) **B.** MPNST cell line as demonstrated by qRT-PCR. \**p*-value <0.05.

- Combined treatment with lysosomotropic agents and BH3-mimetics enhances MPNST cell death.

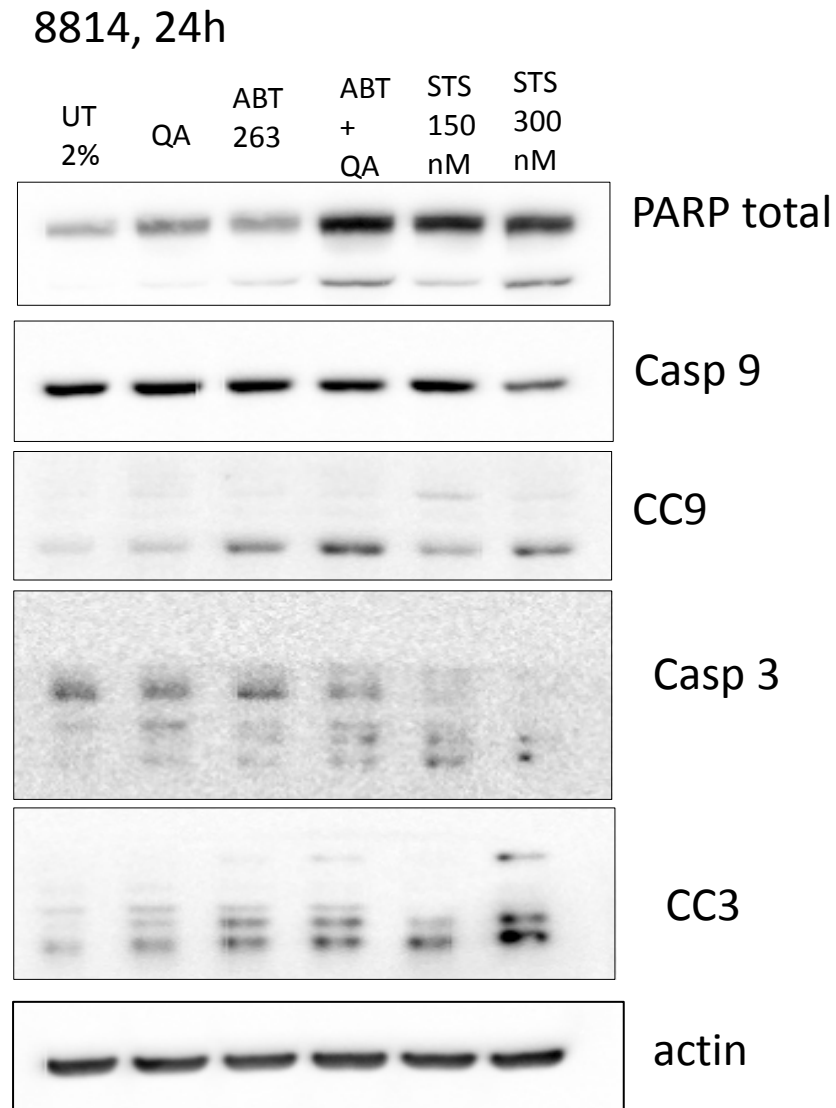
We previously reported that both chloroquine and AT-101 decrease MPNST cell survival. We have extended these observations to examine other lysosomotropic agents, such as quinacrine (QA), and BH3-mimetics, such as ABT-263, and to determine if combinations of these drugs might be more effective in killing MPNST cells *in vitro*. As shown in figure 4, we observed cytotoxic effects of both quinacrine and ABT-263 on MPNST cells and that enhanced cytotoxicity was found if the two drugs were given in combination. Based on these *in vitro* findings, we are now testing these agents alone and in combination in our *in vivo* MPNST transgenic mouse model. This study is underway and will be completed in the final year of the application.



**Figure 4.** Treatment of T265 MPNST cells with the lysosomotropic drug quinacrine (QA) or with the BH3-mimetic ABT 263 results in decreased cell viability (y-axis: fractional cell viability). Combined administration of the two drugs further decreases MPNST cell survival.

- Cytotoxic effects of combined BH3-mimetic and lysosomotropic agents may be due to suppression of USP9X.

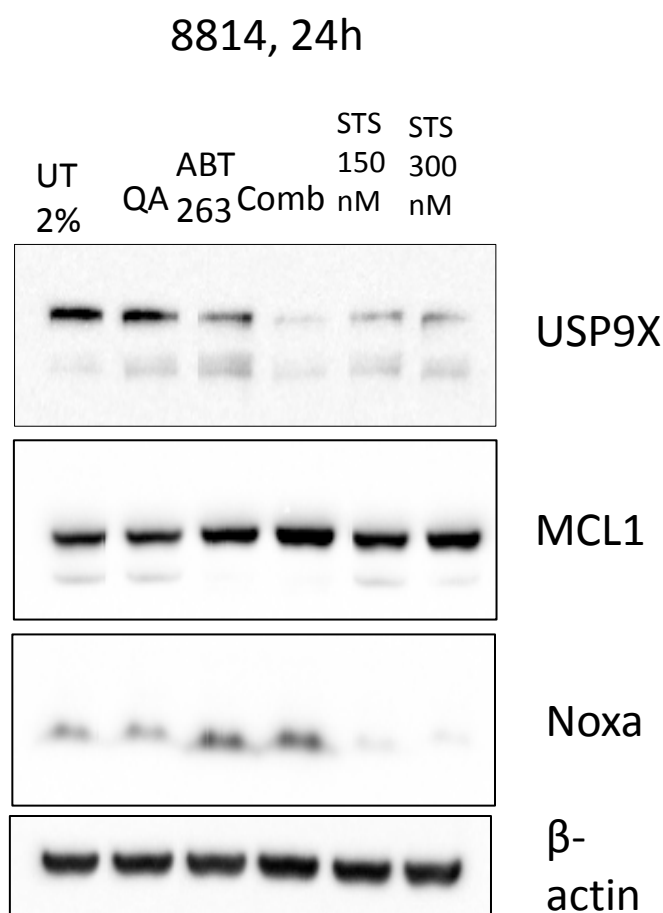
To investigate the mechanisms by which lysosomotropic agents and BH3-mimetics enhance MPNST cell death, we first examined markers of apoptotic cell death by western blot analysis of MPNST cells. As expected from our previous studies, we found a significant increase in cleaved PARP, cleaved caspase-9 and cleaved caspase-3 following treatment of MPNST cells (8814 cell line) with either quinacrine (QA) or ABT-263 (Figure 5). The combination of QA with ABT-263 produced an even more dramatic increase in apoptotic markers.



**Figure 5.** MPNST 8814 cells were treated with QA and ABT-263 alone or in combination for 24 hours and protein extracts were subjected to Western blot analysis for PARP (total is the upper band, cleaved is the lower band), caspase 9 (Casp 9), cleaved caspase 9 (CC9), caspase 3 (Casp 3), cleaved caspase 3 (CC3), and actin (loading control). Staurosporine served as a positive control for apoptosis induction.



To explore the possible molecular mechanism for the pro-apoptotic action of quinacrine and ABT-263, we examined the levels of several Bcl2 family members and USP9X in control and treated MPNST cell lines. USP9X is a deubiquitinating enzyme that plays an important role in regulating the degradation of multiple proteins. USP9X has been implicated in regulating multiple signaling pathways including Notch, Wnt, EGF and mTOR. USP9X has been shown to also regulate stem cell maintenance and it is expressed in the nervous system. Of note, USP9X has been reported to directly affect MCL1 stability which is a key regulator of apoptosis. We treated 8814 and T265 MPNST cells with quinacrine, ABT-263, or the combination of the two drugs for 24 hours and examined levels of USP9X, MCL1, and Noxa (a BH3-only, pro-apoptotic Bcl2 family member). We found that quinacrine had minimal effect on USP9X, MCL1 and Noxa; ABT-263 decreased USP9X and increased Noxa levels, and this effect was markedly enhanced by combined treatment with quinacrine and ABT-263 (Figure 6). Interestingly, levels of MCL1 were relatively unaffected by these treatments suggesting an MCL1-independent cell death pathway.

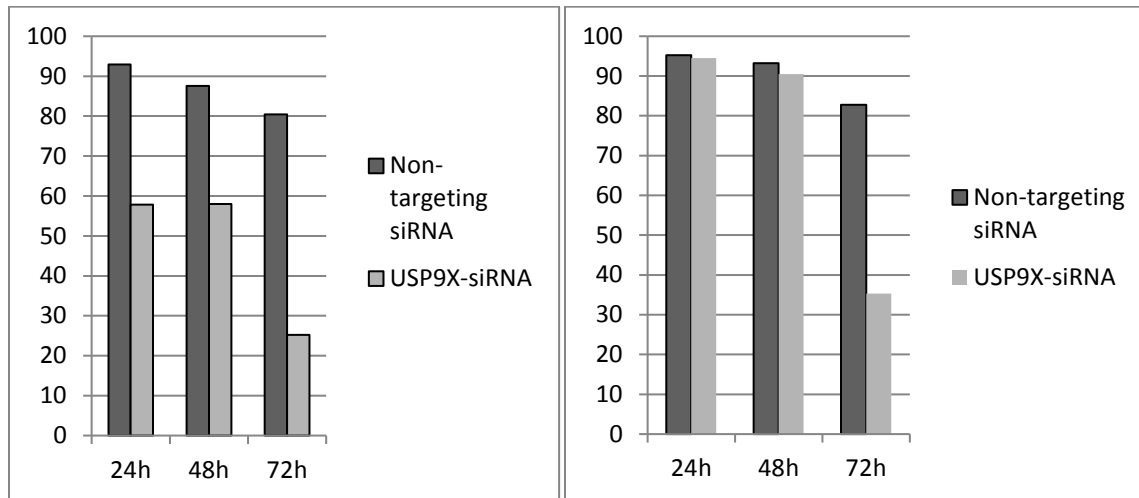


**Figure 6.** MPNST cells (8814) were treated for 24 hours with quinacrine (QA), ABT-263, or combined QA and ABT-263 (Comb) and levels of USP9X, MCL1 and Noxa were measured by Western blot. Staurosporine (STS) served as a positive control for apoptosis induction. Beta-actin is a loading control.

Based upon these observations, we next went on to determine if USP9X might serve as a regulator of MPNST cell death. We tested the effects of USP9X-siRNA versus non-targeting-siRNA treatment on MPNST cell viability over time. In both T265 and 8814 cell lines, we observed a time dependent loss of cell viability following USP9X-siRNA treatment (Figure 7). In particular, the 8814 cell line showed rapid cell death following USP9X knockdown.

### 8814-Viability

### T265-Viability



**Figure 7.** MPNST cells, T265 and 8814, were treated with USP9X targeting or non-targeting siRNA and cell viability (y-axis) was measured over time. Both MPNST cell lines exhibited time-dependent decreased viability after USP9X-siRNA treatment with the 8814 cells showing enhanced sensitivity.

In total, the above studies that we performed over the last year suggest that BH3-mimetics, alone and in combination with lysosomotropic agents, induce MPNST cell apoptosis. In addition, our studies suggest that USP9X may be an important regulator of MPNST cell death although the precise mechanism of death requires additional investigation since our initial studies suggests that it may be independent of MCL1 expression.

**What opportunities for training and professional development has the project provided?**

Dr. Roth met with colleagues at the Pathobiology for Investigators, Students, and Academicians (PISA) Meeting in Houston, TX from October 20-22 and at the Northeast Association of Pathology Chairs Meeting in September, 2016 and informally discussed this work. He was also an invited participant at the European Neuromuscular Centre (ENMC) Workshop in Amsterdam on February 10 and 11, 2017 to discuss quality in translational research for neuromuscular diseases.

**How were the results disseminated to communities of interest?**

Dr. Roth presented lectures on this work at Genentech, San Francisco, CA on September 27, 2016 and to members of the Columbia Translational Neuroscience Initiative on November 4, 2016.

**What do you plan to do during the next reporting period to accomplish the goals?**

In the next reporting period, we will complete our *in vivo* study of the effects of chronic administration of BH3-mimetics (ABT-263) and lysosomotropic agents (quinacrine) on tumor formation and survival in our MPNST transgenic mouse model. We plan to further explore the importance of BH3 mimetic-induced USP9X suppression in regulating MPNST cell viability *in vitro* and attempt to determine if this death pathway is truly independent of MCL1 and other Bcl2 family members. These studies have important implications for understanding how MPNST cell death is regulated and for developing new therapies for patients with neurofibromatosis and MPNSTs.

**4. Impact****What was the impact on the development of the principal discipline(s) of the project?**

BH3 mimetics and lysosomotropic agents are potentially useful new compounds for inhibiting MPNST formation and progression in NF1 patients but they require additional testing in animal models and further definition of their molecular mechanisms of action on MPNST cells. We have laid a solid foundation for these additional studies and we will proceed with the previously proposed *in vitro* and *in vivo* experiments as well as further investigating our novel observation that BH3 mimetics suppress USP9X expression in MPNST cell lines.

**What was the impact on other disciplines?**

Nothing to Report

**What was the impact on technology transfer?**

Nothing to Report

**What was the impact on society beyond science and technology?**

Nothing to Report

**5. Changes/Problems**

Nothing to Report

**6. Products****Publications, conference papers, and presentations****Journal publications:**

Graham CD, Kaza N, Pruitt HC, Gibson LM, Klocke BJ, Shevde LA, Carroll SL, Roth KA. BH3 mimetics suppress CXCL12 expression in human malignant peripheral nerve sheath tumor cells. *Oncotarget*, 2017 Jan 31;8(5):8670-8678. Doi: 10.18632/oncotarget.14398.

**Books or other non-periodical, one-time publications:**

Nothing to Report

**Other publications, conference papers, and presentations:**

Dr. Roth presented lectures on this work at Genentech, San Francisco, CA on September 27, 2016 and to members of the Columbia Translational Neuroscience Initiative on November 4, 2016.

**Website(s) or other Internet site(s)**

Nothing to Report

**Technologies or techniques**

Nothing to Report

**Inventions, patent applications, and/or licenses**

Nothing to Report

**Other Products**

Nothing to Report

**7. Participants & Other Collaborating Organizations****What individuals have worked on the project?**

Kevin A. Roth, MD, PhD – No Change

Elena Bianchetti, Ph.D. – No Change

Hai Tang, M.D. – No Change

Name:	Kevin A. Roth, MD, PhD
Project Role:	Principle Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-0643-995X
Nearest person month worked:	1 Calendar Months
Contribution to Project:	Dr. Roth supervised all aspects of the project.
Name:	Elena Bianchetti, Ph.D
Project Role:	Post Doctoral Researcher
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	3 Calendar Months
Contribution to Project:	Dr. Bianchetti performed all the in vitro tissue culture studies.
Name:	Hai Tang, M.D
Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	1 Calendar Months
Contribution to Project:	Dr. Tang performed all in vivo studies.

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to Report

**What other organizations were involved as partners?**

Nothing to Report

**8. Special Reporting Requirements**

None

**9. Appendices**

None